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# Interactions of (2S,6S;2R,6R)-Hydroxynorketamine, a Secondary Metabolite of (R,S)-Ketamine, with Morphine

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**Abstract:** Ketamine and its primary metabolite norketamine attenuate morphine tolerance by antagonising *N*-methyl-D-aspartate (NMDA) receptors. Ketamine is extensively metabolized to several other metabolites. The major secondary metabolite (2S,6S;2R,6R)-hydroxynorketamine (6-hydroxynorketamine) is not an NMDA antagonist. However, it may modulate nociception through negative allosteric modulation of  $\alpha 7$  nicotinic acetylcholine receptors. We studied whether 6-hydroxynorketamine could affect nociception or the effects of morphine in acute or chronic administration settings. Male Sprague Dawley rats received subcutaneous 6-hydroxynorketamine or ketamine alone or in combination with morphine, as a cotreatment during induction of morphine tolerance, and after the development of tolerance induced by subcutaneous minipumps administering 9.6 mg morphine daily. Tail flick, hot plate, paw pressure and rotarod tests were used. Brain and serum drug concentrations were quantified with high-performance liquid chromatography–tandem mass spectrometry. Ketamine (10 mg/kg), but not 6-hydroxynorketamine (10 and 30 mg/kg), enhanced antinociception and decreased rotarod performance following acute administration either alone or combined with morphine. Ketamine efficiently attenuated morphine tolerance. Acutely administered 6-hydroxynorketamine increased the brain concentration of morphine (by 60%), and brain and serum concentrations of 6-hydroxynorketamine were doubled by morphine pre-treatment. This pharmacokinetic interaction did not, however, lead to altered morphine tolerance. Co-administration of 6-hydroxynorketamine 20 mg/kg twice daily did not influence development of morphine tolerance. Even though morphine and 6-hydroxynorketamine brain concentrations were increased after co-administration, the pharmacokinetic interaction had no effect on acute morphine nociception or tolerance. These results indicate that 6-hydroxynorketamine does not have antinociceptive properties or attenuate opioid tolerance in a similar way as ketamine.

Opioids are widely used to manage severe acute and cancer pain. However, prolonged opioid use in chronic non-cancer pain may have severe drawbacks, including the development of tolerance and dependence. In the United States, eighty per cent of new heroin users have previously misused prescription opioids, and the opioid epidemic has been declared a national emergency [1]. Long-term opioid use may even lead to opioid-induced hyperalgesia that can compromise opioid analgesia and lead to further increases in opioid doses [2]. New approaches are needed to provide safer opioid analgesia with lower doses.

Several *N*-methyl-D-aspartate (NMDA) receptor antagonists have been shown to attenuate opioid tolerance [3–5] mainly via pharmacodynamic interactions. Ketamine, a clinically used NMDA antagonist, produces anaesthesia and also analgesia at lower doses. Clinical evidence supports the perioperative use of low-dose ketamine with morphine to decrease the need for morphine [6], whereas there is lack of evidence to support its use in cancer pain as an adjunct with opioids [7,8].

Ketamine undergoes extensive cytochrome P (CYP)-mediated metabolism to norketamine, hydroxyketamines, dehydronorketamine and hydroxynorketamines [9,10]. The

best characterized metabolite is the main metabolite norketamine, a three to five times weaker NMDA receptor antagonist than ketamine [11–14]. In pre-clinical models, it caused anaesthesia [12], and at lower doses, augmented the effects of morphine in models of thermal, peripheral neuropathic and tonic inflammatory pain [15]. It also inhibited [15] and reversed [16] morphine tolerance. The effects of administering norketamine have not been studied in human beings.

After (R,S)-ketamine administration, (2S,6S;2R,6R)-hydroxynorketamine (6-hydroxynorketamine) is the most abundant hydroxynorketamine found in the plasma and brain of mice [17] and plasma of human beings [18]. It is formed via metabolism from norketamine or hydroxyketamine [19,20]. Its pharmacological properties have been only scarcely studied because initial experiments showed that (R,S)-ketamine and (R,S)-norketamine produced anaesthesia and increased spontaneous locomotor activity during recovery from anaesthesia, whereas 6-hydroxynorketamine had no effects. Therefore, it was considered an inactive metabolite. However, recent studies have shown that 6-hydroxynorketamine is pharmacologically active and it may be responsible for the antidepressant effects of ketamine [17].

The beneficial interaction between ketamine or norketamine and opioids has been considered to derive from pharmacodynamic interactions. However, we have recently reported a pharmacokinetic interaction between both morphine and ketamine

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[21] and morphine and norketamine [16] in the rat. A single dose of ketamine or norketamine administered to rats receiving chronic morphine treatment via subcutaneous pumps caused a manifold increase in the brain and serum concentrations of morphine, an effect due to the inhibition of hepatic UDP-glucuronosyl transferase (UGT)-mediated metabolism of morphine. This finding is supported by previous *in vitro* studies [22,23]. Furthermore, by an unknown mechanism, chronic morphine treatment led to increased ketamine and norketamine brain and serum concentrations after acute administration. Thus, the beneficial effects of ketamine and norketamine in attenuating morphine tolerance may in part be due to a pharmacokinetic interaction. At present, there are no data on the possible pharmacokinetic interactions between opioids and other ketamine metabolites such as 6-hydroxynorketamine.

We hypothesized that 6-hydroxynorketamine may have effects on nociception through either pharmacokinetic or pharmacodynamic interactions. We characterized the effects of 6-hydroxynorketamine on nociception and motor coordination when it was administered alone or combined with morphine in acute and chronic administration settings. Serum and brain drug concentrations were quantified to detect possible pharmacokinetic interactions.

## Methods

**Animals.** Animal care and research procedures were conducted and reported in accordance with the guidelines of The International Association for the Study of Pain [24] and the ARRIVE guidelines [25,26]. The study protocol was approved by the provincial government of Southern Finland (Etelä-Suomen aluehallintovirasto, Hämeenlinna, Finland, ESAVI/7929/04.10.07/2014). Male Sprague Dawley rats (Harlan, Horst, the Netherlands,  $n = 4-7$  per study group; weight 200–250 g) were housed in standard light- and temperature-controlled rooms (lights on 06:00–18:00 hr, temperature  $22 \pm 2^\circ\text{C}$ ) in groups of two in individually ventilated plastic cages. Tap water and standard laboratory chow were available *ad libitum*. All experiments were conducted during the light phase. The animals were habituated to the testing environment for 3 days before the experiments. All behavioural testings were randomized and blinded. The stress and suffering of the experimental animals were minimized, and the animals could freely terminate the noxious stimulation during the tests. After the experiments, the animals were killed by decapitation and tissue samples were collected. A total of 70 rats were used in the experiments.

**Drugs for acute nociceptive tests.** Morphine hydrochloride and racemic ketamine hydrochloride (Ketaminol® vet, 50 mg/ml, Boxmeer, the Netherlands) were purchased from the University Pharmacy, Helsinki, Finland. (2S,6S;2R,6R)-hydroxynorketamine hydrochloride (6-hydroxynorketamine) was purchased from Tocris (Bristol, UK). Morphine and 6-hydroxynorketamine were dissolved, and ketamine was diluted in physiological saline and administered subcutaneously in a volume of 2 ml/kg. All drug concentrations are expressed as free base amounts.

**Opioid tolerance scheme.** Opioid tolerance was induced during 6 days with continuous opioid administration using osmotic minipumps (Alzet 2ML1; Durect, Cupertino, CA, USA). The pumps were filled with 40 mg/ml morphine diluted in sterile water to deliver morphine 9.6 mg/day. For the control animals, sterile water was used

as a vehicle. The pumps were implanted subcutaneously in the back of the rats under isoflurane (3.0%) anaesthesia.

**Behavioural tests.** Tail flick, hot plate and paw pressure tests were used to study heat and mechanical nociception. The rotarod test was used to assess motor coordination. The baseline latencies for each experiment day were measured separately immediately before the administration of any drugs.

Tail flick latencies were measured with a Ugo Basile 37360 (Como, Italy) radiant heat tail flick device. The rats were restrained in hard plastic tubes covered with a dark cloth. After acclimatization, the light was directed to the middle tail at three different points 0.5 cm apart, and the mean of these three values was used as the result. A flick of the tail was considered as thermal nociception terminating the test. If an individual measurement reached cut-off (10 sec.), no further tests were performed at that time-point. The intensity of the device was adjusted to produce a baseline latency of approximately 4 sec.

Hot plate tests were performed with a Ugo Basile 35100 hot/cold plate device. The rats were put into a circular transparent plastic cage on the hot plate ( $52 \pm 0.2^\circ\text{C}$ ). The latency to the first thermal nociceptive reaction, such as licking or shaking of the hind paw or jumping, was measured. A cut-off time of 60 sec. was used.

Paw pressure tests were performed with a Ugo Basile 37215 device. In the test, animals were gently wrapped in a towel and the left hind paw of the rat was placed under a pivot. The force applied to the paw was gradually increased up to a cut-off of 500 g. A brisk withdrawal of the paw or vocalization terminated the measurement. The test was performed twice at each time-point with a 1-min interval, and the mean of these two tests was used as the result.

Rotarod tests were performed with a Ugo Basile 47700 rat rotarod apparatus, diameter 70 mm, with the speed accelerating from 10 to 40 r.p.m. during the course of 60 sec. The rat was placed on the rotating rod, and the time it stayed on it was measured, with a cut-off time of 60 sec.

At each time-point, the tests were performed in the same order. The tail flick test was performed first and the hot plate test second, followed by the paw pressure test after a pause of 1 min. The rotarod test was performed last.

**Determination of drug and metabolite concentrations.** For concentration measurements, the rats were decapitated and whole brain and blood samples were collected. Brain samples were covered in aluminium foil and snap-frozen in liquid nitrogen, after which they were stored at  $-80^\circ\text{C}$ . The blood samples were let to coagulate on ice after which they were centrifuged at  $2000 \times g$  for 10 min. at  $+4^\circ\text{C}$ . Serum was collected and stored at  $-80^\circ\text{C}$ . For concentration measurements, the whole brain samples were weighed, homogenized and diluted in sterile water. Brain tissue samples from untreated rats were individually spiked with the studied compounds and used as a standard. The measurements were carried out as previously described [27] with some modifications using an Agilent 1100 series HPLC system (Agilent Technologies, Waldbronn, Germany) coupled to an API 3000 tandem mass spectrometry (AB Sciex, Toronto, ON, Canada), and the chosen method was validated also for the analysis of ketamine, norketamine and 6-hydroxynorketamine (data not shown). The chromatographic separation of morphine, morphine-3-glucuronide (M3G), ketamine, norketamine and 6-hydroxynorketamine was achieved on Atlantis HILIC Silica column (3  $\mu\text{m}$  particle size,  $2.1 \times 100$  mm I.D.) (Waters, Milford, MA, USA) using a gradient elution of mobile phase consisting of acetonitrile and 20-mmol/l ammonium acetate (pH 3.0, adjusted with formic acid). An aliquot (5  $\mu\text{l}$ ) was injected at a flow rate of 200  $\mu\text{l}/\text{min}$  to give a total chromatographic run time of 24 min. Oxycodone-d3 (Cerilliant, Round Rock, TX, USA) served as an internal standard for morphine

and M3G, and 6-hydroxynorketamine served as its own internal standard. Deuterium-labelled internal standards (ketamine-d4 and norketamine-d4; Cerilliant) were used for ketamine and norketamine. The target ion transitions monitored were as follows: morphine *m/z* 286–152, M3G *m/z* 462–286, ketamine *m/z* 238–125, norketamine *m/z* 224–125 and 6-hydroxynorketamine *m/z* 240–141. The limit of quantification for morphine, M3G and 6-hydroxynorketamine was 1.0 ng/ml in serum and 5.0 ng/g in brain, and 0.5 ng/ml for ketamine and norketamine in serum and 2.5 ng/g in brain. The calibration curves were linear over the concentration range of LOQ–250 ng/ml, and day-to-day coefficients of variation were below 15% at relevant concentrations for all analytes. None of the measured compounds interfered with the mass spectrometric assay.

*Effects of 6-hydroxynorketamine on nociception and motor coordination alone and in co-administration with morphine.* The effects of subcutaneous 6-hydroxynorketamine on nociception and motor coordination were studied alone at two doses (10 and 30 mg/kg). These doses were selected because in a previous study [17], the dose of 10 mg/kg showed significant antidepressant effects in a mouse model. After a week of washout, the effects of 6-hydroxynorketamine on morphine antinociception were studied using the same animals. Subcutaneous morphine (2.5 mg/kg) was administered 15 min. before 6-hydroxynorketamine (10 or 30 mg/kg). Tail flick, hot plate, paw pressure and rotarod tests were performed for up to 90 min. after 6-hydroxynorketamine administration, with an additional rotarod measurement at 10 min. In all studies, subcutaneous ketamine (10 mg/kg) was used as a positive control drug.

*Effects of acute 6-hydroxynorketamine in rats under chronic morphine administration.* We investigated the ability of 6-hydroxynorketamine to reverse the established morphine tolerance. On day 0, rats received either morphine or vehicle pumps. Development of antinociceptive tolerance was confirmed on day 6, after which subcutaneous 6-hydroxynorketamine (10 or 30 mg/kg) was administered. Ketamine 10 mg/kg was used as a positive control. Nociception was monitored at 30-min. intervals using the tail flick, hot plate and paw pressure tests. Rotarod performance was measured additionally also at 10 min. after acute drug administration. After the behavioural tests at 90 min., rats were decapitated and tissues were gathered for drug concentration measurements.

*Effects of 6-hydroxynorketamine in developing morphine tolerance.* This experiment assessed the possible effects of 6-hydroxynorketamine in the development of morphine antinociceptive tolerance. Before the implantation of morphine minipumps, rats were given either subcutaneous 6-hydroxynorketamine (20 mg/kg) or vehicle. This treatment continued twice daily until the end of day 5. The nociceptive behaviour of the rats was monitored before the administration of any drugs (day 0) and in the morning of days 2, 4 and 6. The measurements were performed before the administration of the morning 6-hydroxynorketamine dose. On day 6, a single dose of subcutaneous morphine (10 mg/kg) was administered to both groups and the effects were monitored at 30 and 90 min. after administration. Finally, rats were decapitated and tissue samples collected.

*Statistical analysis.* The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology [28] with minor exceptions. All experiments were performed blinded, but the data analysis was performed unblinded. In some data points (concentration analyses), the number of samples per group was four. However, all nociceptive behavioural experiments had six to seven animals in the group. The results of the tail flick, hot plate and paw pressure tests are expressed as percentage of the

maximum possible effect (MPE%), calculated as  $MPE\% = [(postdrug\ latency - baseline\ latency)/(cut-off\ time - baseline\ latency)] \times 100\%$ , which takes into account the differences in baseline nociceptive latencies. The results are presented as means of the groups ( $\pm$ S.E.M.). The behavioural data were tested for statistically significant differences in the mean values by two-way analysis of variance (ANOVA), and a Holm–Sidak *post hoc* analysis was performed. For the concentration data, an unpaired two-tailed *t*-test or one-way ANOVA followed by the Holm–Sidak *post hoc* analysis was used. For the nonparametric rotarod test data, the Kruskal–Wallis test followed by Dunn's multiple comparison was used. The difference was considered significant at  $p < 0.05$  in both the analysis of variance and the *post hoc* test. The data were analysed using GRAPHPAD PRISM, version 6.0e, for Mac OS X (GraphPad Software, La Jolla, CA, USA).

## Results

*6-hydroxynorketamine does not possess antinociceptive properties in acute thermal or mechanical pain.*

Compared with vehicle, acutely administered subcutaneous ketamine (10 mg/kg) caused small but significant antinociception in the tail flick (fig. 1A) and paw pressure (fig. 1C) tests but not in the hot plate test (fig. 1B). Ketamine also significantly impaired motor coordination in the rotarod test at 10 min. after administration (fig. 1D). The administered doses of 6-hydroxynorketamine (10 and 30 mg/kg) did not have effects on antinociception or motor coordination at any time-point.

*Ketamine but not 6-hydroxynorketamine augments acute morphine antinociception.*

Rats received subcutaneous morphine (2.5 mg/kg) and 15 min. later subcutaneous ketamine (10 mg/kg) or 6-hydroxynorketamine at two doses (10 and 30 mg/kg). Morphine antinociception was at its greatest at 45 min. after administration, and ketamine significantly augmented the effect of morphine in all the nociceptive tests (fig. 2A–C). The motor coordination of rats that received ketamine and morphine was significantly compromised 10 min. after ketamine administration. Co-administration of 6-hydroxynorketamine did not significantly augment morphine antinociception or decrease motor coordination.

*Development of morphine tolerance under 6 treatment days.*

Morphine pumps were implanted on day 0. Robust antinociception was detected one day after the procedure in all the nociceptive tests (fig. 3A–C). On day 6, tolerance to the antinociceptive effect of morphine was fully developed.

*Attenuation of morphine tolerance by ketamine, but not 6-hydroxynorketamine.*

Upon the confirmation of the development of morphine tolerance on day 6, rats received acute subcutaneous ketamine (10 mg/kg) or 6-hydroxynorketamine (10 or 30 mg/kg). In the tail flick test (fig. 4A), ketamine reversed morphine tolerance from 60 min. after administration to the end of the measurement period (90 min.). Ketamine induced significant reversal of tolerance also in the hot plate test (fig. 4B) and paw

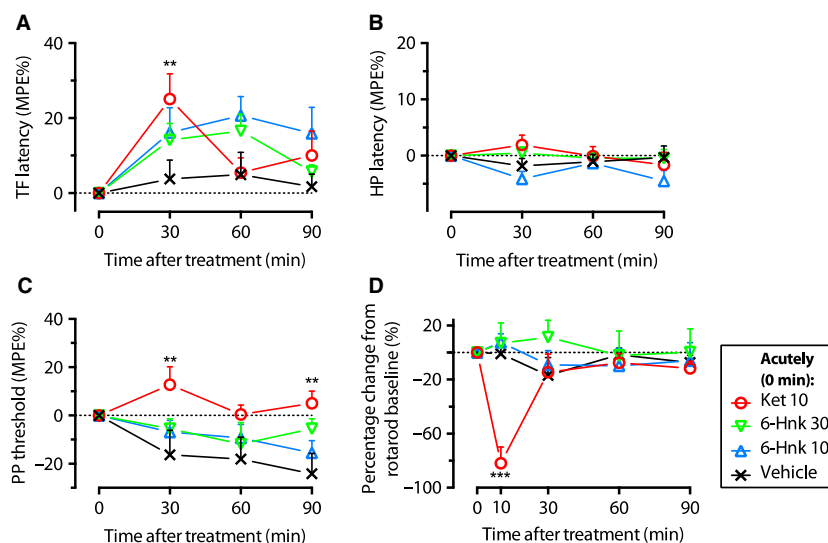


Fig. 1. Effects of acute ketamine or 6-hydroxynorketamine on nociception and motor coordination. Drug-naïve rats received subcutaneous (2S,6S;2R,6R)-hydroxynorketamine (6-hydroxynorketamine) 30 mg/kg (6-Hnk 30) or 10 mg/kg (6-Hnk 10), ketamine 10 mg/kg (Ket 10) or vehicle. Nociceptive thresholds were monitored up to 90 min. after administration using the tail flick (TF, A), hot plate (HP, B) and paw pressure (PP, C) tests. The rotarod test (D) was used to screen for motor coordination deficiencies. The mean ( $\pm$ S.E.M.) of the maximum possible effect (MPE%) is plotted for the nociceptive tests, and for the rotarod test, the mean ( $\pm$ S.E.M.) percentage change from the baseline is shown.  $**p < 0.01$ ,  $***p < 0.001$  as compared with the group that received vehicle.  $n = 7$  per group.

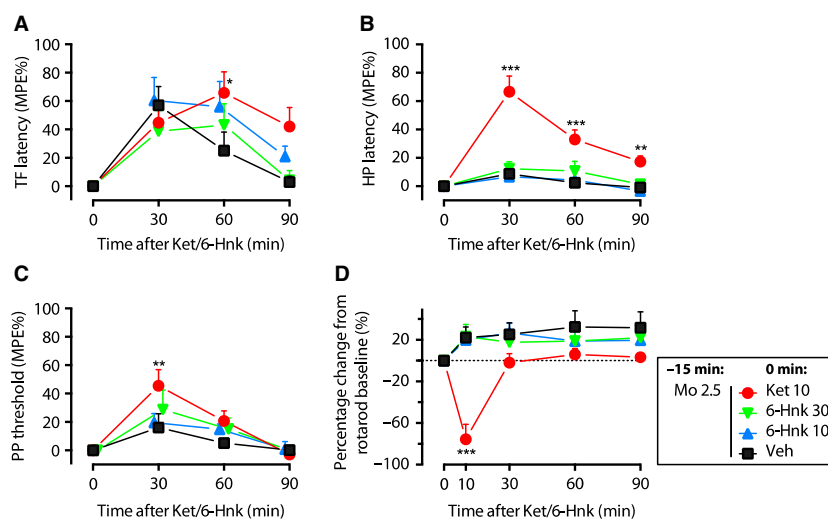


Fig. 2. Effects of ketamine or 6-hydroxynorketamine on acute morphine nociception and motor coordination. All rats received subcutaneous morphine (Mo) 2.5 mg/kg at -15 min. time-point. At time-point 0, rats received subcutaneous (2S,6S;2R,6R)-hydroxynorketamine (6-hydroxynorketamine) 30 mg/kg (6-Hnk 30) or 10 mg/kg (6-Hnk 10), ketamine 10 mg/kg (Ket 10) or vehicle. Nociceptive thresholds were monitored up to 90 min. after administration using the tail flick (TF, A), hot plate (HP, B) and paw pressure (PP, C) tests. The rotarod test (D) was used to screen for motor coordination deficiencies. The mean ( $\pm$ S.E.M.) of the maximum possible effect (MPE%) is plotted for the nociceptive tests, and for the rotarod test, the mean ( $\pm$ S.E.M.) percentage change from the baseline is shown.  $**p < 0.01$ ,  $***p < 0.001$  as compared with the group that received morphine with vehicle.  $n = 7$  per group.

pressure test (fig. 4C) at 30 and 60 min. after administration. Administration of 6-hydroxynorketamine did not lead to attenuation of morphine tolerance during the 90-min. measurement period.

At 90 min., after ketamine or 6-hydroxynorketamine administration, serum and brain samples were obtained. Acutely administered 6-hydroxynorketamine (30 mg/kg) significantly increased (60%) the morphine brain concentration (fig. 4D).

The difference in serum concentrations was parallel but did not reach significance. The serum M3G:morphine metabolic ratios were  $7.9 \pm 1.2$  and  $6.1 \pm 0.8$  for the vehicle- and 6-hydroxynorketamine-treated groups, respectively ( $p = 0.22$ ), indicating no significant inhibition of morphine metabolism by 6-hydroxynorketamine. At 90 min. after administration, notable 6-hydroxynorketamine concentrations were observed both in the brain and serum (fig. 4E) with a brain-to-serum ratio of



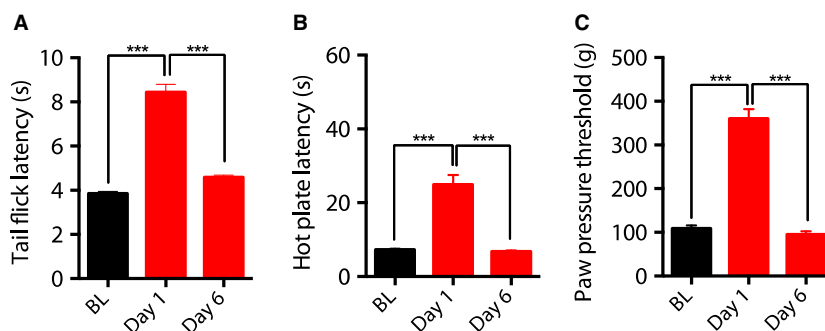


Fig. 3. Tolerance development to the antinociceptive effect of morphine delivered by subcutaneous minipumps. On day 0, s.c. minipumps delivering morphine 9.6 mg/day were implanted under brief isoflurane anaesthesia. The means ( $\pm$ S.E.M.) of the tail flick latencies (A), hot plate latencies (B) and paw pressure thresholds (C) are shown 1 and 6 days after pump implantation. The mean raw latencies or thresholds are shown to allow assessment of data quality. \*\*\* $p < 0.001$ ; significantly different between the indicated groups.  $n = 28$ .

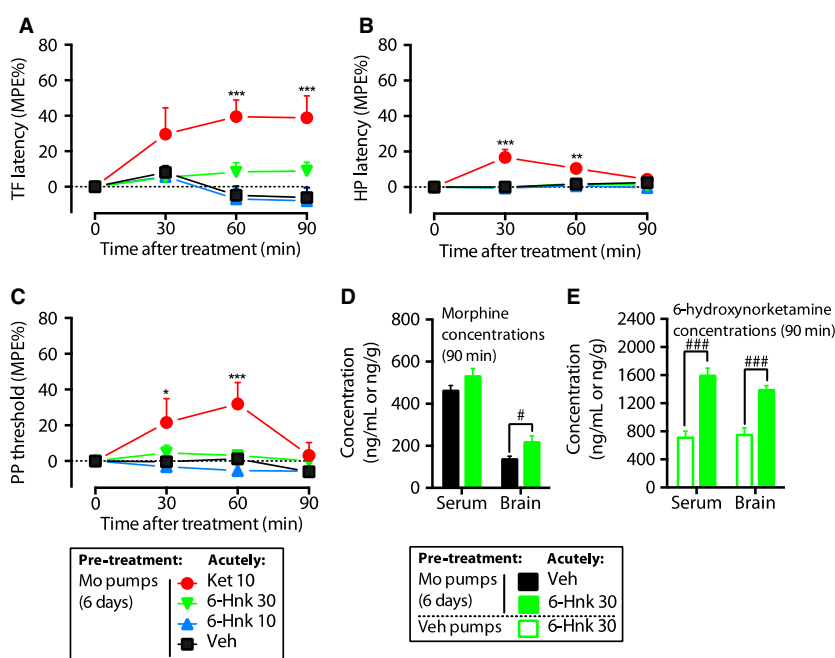


Fig. 4. Effects of acute ketamine or 6-hydroxynorketamine on nociception in morphine-tolerant rats under chronic morphine treatment. On day 0, rats were implanted with subcutaneous minipumps administering morphine 9.6 mg/day. On day 6, rats received subcutaneous (2S,6S;2R,6R)-hydroxynorketamine (6-hydroxynorketamine) 30 mg/kg (6-Hnk 30) or 10 mg/kg (6-Hnk 10), ketamine 10 mg/kg (Ket 10) or vehicle. Nociceptive thresholds were monitored up to 90 min. after administration using the tail flick (TF, A), hot plate (HP, B) and paw pressure (PP, C) tests. The mean ( $\pm$ S.E.M.) of the maximum possible effect (MPE%) is plotted for the nociceptive tests. At 90 min., the mean serum and whole brain concentrations of morphine (D) and 6-hydroxynorketamine (E) in selected groups were quantified using HPLC-MS. Mean concentrations ( $\pm$ S.E.M.) are shown. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  as compared with the group that received morphine with vehicle. # $p < 0.05$ , ### $p < 0.001$  between the indicated groups.  $n = 7$  per group.

1.1. Pre-treatment with morphine pumps led to doubled 6-hydroxynorketamine concentrations both in the brain and serum. No significant amounts of ketamine or norketamine were detected after the administration of 6-hydroxynorketamine.

#### *Co-administration of 6-hydroxynorketamine with chronic morphine does not lead to reduced development of tolerance.*

Rats were implanted with morphine pumps on day 0, and subcutaneous vehicle or 6-hydroxynorketamine (20 mg/kg) was administered twice daily. Nociceptive latencies were monitored

on days 2, 4 and 6 before the administration of 6-hydroxynorketamine or vehicle. On day 2, morphine antinociception was at its highest in the tail flick test (fig. 5A), whereas in the hot plate (fig. 5B) and paw pressure (fig. 5C) tests, tolerance to the effect of morphine had already developed. On day 2 in the hot plate test, compared with vehicle, co-administration of 6-hydroxynorketamine led to a significantly greater nociceptive latency. However, this effect was not observed in the tail flick or paw pressure tests. On day 6, after baseline measurements, rats received a subcutaneous test dose of morphine (10 mg/kg) without any acute 6-hydroxynorketamine. Morphine produced

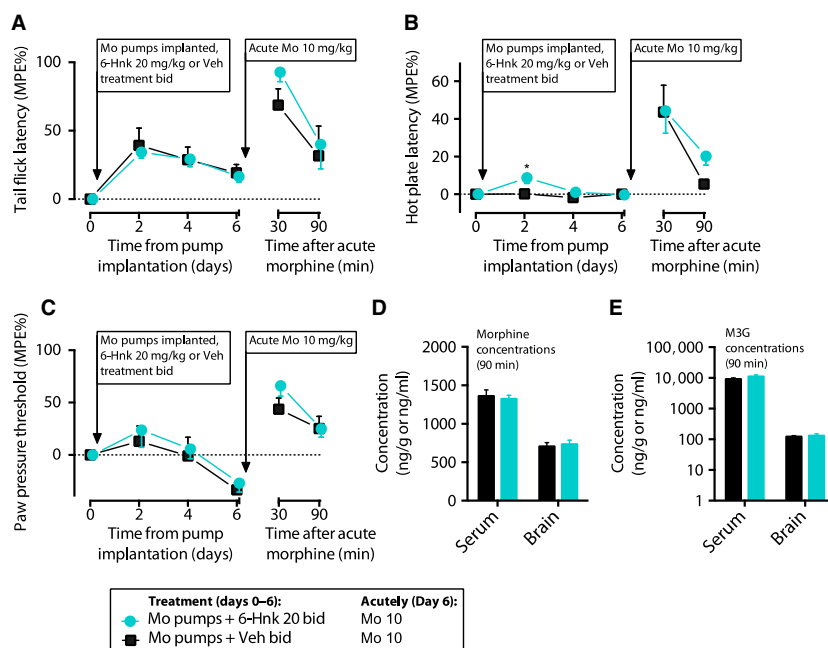


Fig. 5. Effects of 6-hydroxynorketamine cotreatment on developing morphine tolerance. On day 0, rats were implanted with subcutaneous morphine (Mo) pumps delivering 9.6 mg/day morphine. During days 0–5, rats received subcutaneous (2S,6S;2R,6R)-hydroxynorketamine (6-hydroxynorketamine) 20 mg/kg (6-Hnk 20) or vehicle (Veh) twice daily (bid). The last injection was given on day 5 evening. The development of antinociceptive tolerance to morphine was assessed on days 2, 4 and 6 using tail flick (TF, A), hot plate (HP, B) and paw pressure (PP, C) tests. In the morning of day 6, after assessment of the baseline nociceptive values, both groups received an acute subcutaneous dose of morphine (10 mg/kg) and nociceptive tests were used 30 and 90 min. after administration. The mean ( $\pm$ S.E.M.) of the maximum possible effect (MPE%) is plotted for the nociceptive tests. Directly after the 90-min. measurements, serum and whole brain samples were collected and the mean ( $\pm$ S.E.M.) morphine (D) and morphine-3-glucuronide (M3G, E) concentrations were quantified using HPLC-MS. \* $p < 0.05$  as compared with the group that received morphine pumps and vehicle.  $n = 6$ –7 per group.

significant antinociception in all nociceptive tests 30 min. after administration. Previous cotreatment with 6-hydroxynorketamine did not significantly influence the acute effects of the test dose, and also no differences in serum or brain concentrations of morphine (fig. 5D) or M3G (fig. 5E) were found.

## Discussion

The present study shows that acute administration of ketamine significantly enhanced morphine antinociception, whereas its secondary metabolite 6-hydroxynorketamine did not influence nociceptive latencies alone or co-administered with morphine. Developed morphine tolerance was also attenuated by ketamine but not 6-hydroxynorketamine. Co-administration of 6-hydroxynorketamine twice daily did not affect the development of morphine tolerance. Acute 6-hydroxynorketamine did not impair motor coordination, in agreement with previous results [17].

Significant 6-hydroxynorketamine brain concentrations (800 ng/g  $\approx$  3  $\mu$ M) were detected 90 min. after the administration of a 30 mg/kg dose, supporting previous findings showing that 6-hydroxynorketamine readily passes to the central nervous system [12,29]. This exceeds the  $EC_{50}$  for negative allosteric modulation of  $\alpha 7$ -nACh receptors (100 nM) [30]. In the present and also in our previous study, ketamine

(10 mg/kg s.c.) administration significantly attenuated morphine tolerance at 30 and 90 min. At these time-points, 6-hydroxynorketamine brain concentrations were  $770 \pm 370$  and  $660 \pm 300$  ng/g, respectively (mean  $\pm$  S.D.,  $n = 4$ , unpublished findings). As the 6-hydroxynorketamine brain concentrations were at the same level after ketamine or direct 6-hydroxynorketamine administration in those studies, we can conclude that 6-hydroxynorketamine does not contribute to the effects of ketamine on morphine tolerance.

Effects of 6-hydroxynorketamine on nociception have not been previously studied. The original work studying the anaesthetic properties of ketamine, norketamine and 6-hydroxynorketamine [12] found no anaesthetic effects by 6-hydroxynorketamine. Later studies confirmed that 6-hydroxynorketamine is not an NMDA receptor antagonist [30]. However, 6-hydroxynorketamine is a negative allosteric modulator of  $\alpha 7$ -nACh receptors already at 100 nM [30], a concentration much lower than the one achieved in this study after the 30 mg/kg dose (3  $\mu$ M). Negative allosteric modulation of  $\alpha 7$ -nACh receptors proposedly leads to multiple effects that may have opposite actions regarding nociception and opioid tolerance. Firstly, inhibition of  $\alpha 7$ -nACh receptor function and decreased cellular  $Ca^{2+}$  influx in nociceptors may lead to decrease in nociceptive glutamatergic signalling. Secondly, activation of the downstream mechanistic target of rapamycin (mTOR) pathway [31] by 6-hydroxynorketamine may lead to

decreased production of D-serine [31–33], an important NMDA receptor co-agonist. Decreased D-serine levels have previously been associated with attenuation of morphine tolerance [34,35]. However, in other studies, activation of the mTOR pathway has been linked to the development of opioid tolerance and hyperalgesia [36,37]. Interestingly,  $\alpha$ 7-nACh receptor agonists have shown efficacy in pre-clinical models of neuropathic pain [38,39] and remifentanyl-induced hyperalgesia [40] by reducing the release of glial pro-inflammatory cytokines. Finally, recent studies exploring the mechanisms of 6-hydroxynorketamine-induced antidepressive actions show that 6-hydroxynorketamine may indirectly increase excitatory  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor glutamatergic signalling [17,41]. Based on these observations, it is difficult to predict the net effect of  $\alpha$ 7-nACh receptor antagonists such as 6-hydroxynorketamine on opioid antinociception. In the present study, administration of 30 mg/kg 6-hydroxynorketamine resulted in significant brain concentrations but did not produce either antinociception or pronociception alone or combined with morphine in acute thermal and mechanical tests of nociception. Moreover, 6-hydroxynorketamine did not attenuate developed opioid tolerance in a similar manner as acute ketamine did. Also, the chronic twice-daily administration of 6-hydroxynorketamine did not affect the development of tolerance.

In the tolerance experiment of the present study, acutely administered 6-hydroxynorketamine increased the brain morphine concentration by 60% with small (non-significant) additive effects (15 MPE%) in the nociceptive tests compared with the group that received vehicle. It is likely that in well-developed morphine tolerance, a 60% increase in the brain morphine concentration is not sufficient to cause significant attenuation of tolerance. In our previous studies using a similar model of morphine tolerance, acute ketamine (10 mg/kg) [21] and norketamine (30 mg/kg) [16] administered to rats under chronic morphine treatment increased brain and serum morphine concentrations much more, three- to fivefold 90 min. after acute administration. Previous *in vitro* studies show that ketamine inhibits UGT2B7 [22,23], an enzyme that metabolises morphine to its glucuronides [42]. In rats, morphine is glucuronidated to M3G by the orthologue Ugt2b1 [43–45]. In our study, the increase in morphine concentrations was associated with a decrease in the serum and liver M3G-to-morphine metabolic ratio [21], indicating the inhibition of morphine metabolism by ketamine and norketamine. So far, no data about the inhibitory properties of UGT2B7 and/or Ugt2b1 by hydroxynorketamines exist. The present results suggest that in the rat, 6-hydroxynorketamine is a much weaker inhibitor of the Ugt2b1 enzyme than ketamine.

Chronic administration of morphine led to doubled 6-hydroxynorketamine concentrations in the brain 90 min. after acute administration, which is an important observation for future drug development. A similar interaction was also found when ketamine [21] and norketamine [16] were administered to morphine-treated rats. The mechanism behind this interaction needs further study.

In conclusion, this study strengthens the pre-clinical evidence that ketamine efficiently augments morphine antinociception and reverses morphine tolerance. The secondary metabolite of ketamine, (2S,6S;2R,6R)-hydroxynorketamine, did not show antinociceptive effects in thermal or mechanical models of nociception when administered either alone or with acute or chronic morphine. It also did not affect motor coordination of rats. Acute 6-hydroxynorketamine only slightly increased the brain concentrations of morphine, suggesting that it is not a strong inhibitor of morphine glucuronidation. The effects of ketamine metabolites should be studied also in models of neuropathic pain to better understand the mechanisms of ketamine and its metabolites in the treatment of pain.

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#### Conflict of Interest

Pekka Rauhala is a medical adviser for Orion Pharma, Espoo, Finland.

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